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Angela Cadou,[*†#] Anne Couturier,[*†] Cathy Le Goff,[*†] Teresa Soto,[‡] Ida Miklos,[§] Matthias Sipiczki,[§] Linfeng Xie,[|] James R. Paulson,[|] Jose Cansado,[‡] and Xavier Le Goff[*†¶]

[*]CNRS UMR6061 Institut de Génétique et Développement de Rennes, France.

[†]Université de Rennes 1, UEB, IFR140, Rennes, France.

[‡]Department of Genetics and Microbiology, Facultad de Biología, Universidad de Murcia, 30071 Murcia, Spain.

[§]Department of Genetics and Applied Microbiology, University of Debrecen, H-4032 Debrecen, Hungary.

[|]Department of Chemistry, University of Wisconsin-Oshkosh, Oshkosh, WI 54901, U.S.A.

[#]present address: Département de Biochimie, Université de Lausanne, 1066 Epalinges, Switzerland

[¶]To whom correspondence should be addressed: CNRS UMR6061 Institut de Génétique et Développement de Rennes, Faculté de Médecine, 2 avenue du Professeur Léon Bernard 35043 Rennes Cedex, France ; Tel: +33 2 23 23 45 27 ; FAX: +33 2 23 23 44 78 ; email : xavier.le-goff@univ-rennes1.fr

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Abbreviations: 1NM-PP1, 4-Amino-1-*tert*-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine; BFA, Brefeldin A; CWI, Cell Wall Integrity; DMSO, dimethyl sulfoxide.

ABSTRACT

Cell morphogenesis is a complex process that depends on cytoskeleton and membrane organization, intracellular signalling and vesicular trafficking. The rod shape of the fission yeast *Schizosaccharomyces pombe* and the availability of powerful genetic tools make this species an excellent model to study cell morphology. Here we have investigated the function of the conserved Kin1 kinase. Kin1-GFP associates dynamically with the plasma membrane at sites of active cell surface remodeling and is present in the membrane fraction. *Kin1*Δ null cells show severe defects in cell wall structure and are unable to maintain a rod shape. To explore Kin1 primary function, we constructed an ATP analog-sensitive allele *kin1-as1*. Kin1 inhibition primarily promotes delocalization of plasma membrane-associated markers of actively growing cell surface regions. Kin1 itself is depolarized and its mobility is strongly reduced. Subsequently amorphous cell wall material accumulates at the cell surface, a phenotype that is dependent on vesicular trafficking, and the Cell Wall Integrity (CWI) Mitogen Activated Protein Kinase (MAPK) pathway is activated. Deletion of CWI MAPK components reduces *kin1*Δ hypersensitivity to stresses such as those induced by Calcofluor white and SDS. We propose that Kin1 is required for a tight link between the plasma membrane and the cell wall.

INTRODUCTION

The control of cell morphology is necessary for the correct execution of many cellular functions as well as for morphogenetic transitions during the life cycle. In metazoan organisms cell morphogenesis involves diverse mechanisms including cell adhesion, migration and proliferation. In addition, establishment and maintenance of cell morphology requires a complex coordination between cytoskeleton and plasma membrane organization, cell polarity and intracellular signalling cascades. Unicellular eukaryotes such as yeast constitute excellent model organisms to identify and study the regulatory mechanisms of cell morphogenesis.

Among yeasts, the fission yeast *Schizosaccharomyces pombe* is a powerful model because cells exhibit a strict rod shape during their vegetative life cycle. The cell diameter remains constant and cells extend at only one cell end during early G2 but at both cell ends later in G2. This switch to a bipolar growth pattern is called “New End Take Off” (NETO). During interphase, microtubules (MTs) are organized along the main cell axis as longitudinal bundles with an anti-parallel configuration. The MT plus ends extend towards the cell ends where they contribute to the delivery of polarity factors at the cortex whereas the minus ends are in close contact with the nucleus (for reviews, La Carbona *et al.*, 2006; Sawin and Tran, 2006). During polarized growth, cell wall remodeling resumes in the cell ends where F-actin cables and patches are polymerized. The F-actin cytoskeleton shows a spatial and temporal relationship with proteins of the endocytic machinery (Galletta and Cooper, 2009). For3 is a formin responsible for the nucleation of interphase F-actin cables onto which exocytic vesicles are delivered (Feierbach and Chang, 2001). At mitotic onset, F-actin is reorganized with several other proteins as a contractile ring in the middle of the cell and cytoplasmic microtubules are depolymerized while a mitotic spindle is assembled within the nucleus.

At the plasma membrane, vesicular trafficking involves a balance between exocytosis and endocytosis. Several proteins regulate this balance. Molecular motors such as the type V myosin Myo4 are required for targeting of vesicles and cell wall-synthesizing enzymes to the plasma membrane along F-actin cables (Feierbach and Chang, 2001; Motegi *et al.*, 2001; Mulvihill *et al.*, 2006; Win *et al.*, 2001). Sla2 is a transmembrane protein that regulates endocytosis and F-actin organization and controls plasma membrane internalization (Castagnetti *et al.*, 2005; Iwaki *et al.*, 2004). The *psy1* gene encodes a syntaxin 1 homolog, a component of the docking/fusion system t-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), and is involved in sporulation mechanisms. Psy1 is essential for vegetative growth and is localized at the plasma membrane during the vegetative cell cycle, including the invaginating membranes during septum synthesis (Nakamura *et al.*, 2001).

Fission yeast cell shape also depends on the composition and the polarized synthesis of the cell wall, a complex, rigid and dynamic polysaccharide structure that overlays the plasma membrane (Perez and Ribas, 2004). This structure is composed of cross-linked polysaccharides and glycoproteins. An essential function of the cell wall is to provide mechanoresistance to abrupt changes in environmental conditions. Cell wall synthesis and remodeling during the cell cycle are tightly regulated and require oriented intracellular trafficking to target cell wall-synthesizing enzymes to polarized growth sites. As a consequence, cell wall structure could be affected by mutants for functions in transport, trafficking, glycosylation or cell cycle control. In *S. pombe,* various glucan synthases have been identified. For example, Ags1 is necessary for α1,3-glucan synthesis (Hochstenbach *et al.*, 1998; Katayama *et al.*, 1999). Bgs1, Bgs2, Bgs3 and Bgs4 are the β1,3-glucan synthases involved, respectively, in septum formation (Cortes *et al.*, 2002; Liu *et al.*, 2002), cell sporulation (Martin *et al.*, 2000), elongation (Martin *et al.*, 2003) and cell wall growth and

prevention of cell lysis during cytokinesis (Cortes *et al.*, 2005). The stability of Bgs1 at the plasma membrane depends on the regulatory factor Cfh3 (Sharifmoghadam and Valdivieso, 2009).

Fission yeast morphology mutants have been isolated using genetic screens. Molecular cloning of the corresponding genes has revealed distinct regulatory mechanisms involving cell wall assembly, F-actin polarization, polarity factors and interphase microtubule functions. Some mutants such as those of the *orb* class are unable to establish a rod shape whereas another group called *tea* mutants are defective in restricting opposite growth zones (for a review, Hayles and Nurse, 2001). Yeast mutants defective in general cell wall metabolism also show extensive cell shape defects (for a review see Ishiguro, 1998).

Mitogen Activated Protein Kinase (MAPK) signalling cascades convert a stimulus detected at the cell surface (after a change in environmental conditions) to an intracellular signal to promote an adapted cellular response (Waskiewicz and Cooper, 1995). MAPK cascades are composed of a core kinase module which includes a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. The MAPK pathway is activated by upstream regulators such as protein kinases, small Rho GTPases and transmembrane protein sensors. Activation of MAPK promotes a transcriptional response as well as cytoplasmic responses such as cytoskeletal reorganization and cell cycle delay. In fission yeast, three MAPK signalling cascades have been described. These include the mating pheromone-responsive Spk1 pathway, the stress-activated protein kinase (SAPK) Sty1 pathway and the Cell Wall Integrity (CWI) Pmk1 pathway. When the cell wall is damaged, for instance by chemicals or by abrupt modification of the osmotic pressure, the CWI MAPK pathway is activated (Barba *et al.*, 2008; Levin, 2005). The fission yeast CWI pathway is composed of the MAPKKK Mkh1, the MAPKK Pek1 and the MAPK Pmk1 (Loewith *et al.*, 2000; Madrid *et al.*, 2006; Sengar *et al.*, 1997; Sugiura *et al.*, 1999; Toda *et al.*, 1996; Zaitsevskaya-Carter

and Cooper, 1997). Ultimately, the transcription factor Atf1 is a major downstream Pmk1 target for stress response (Takada *et al.*, 2007). Pmk1 activity is also dependent on the cell cycle and peaks at the time of cell separation during cytokinesis (Madrid *et al.*, 2007). Fission yeast CWI components are detected within the cytoplasm and the nucleus throughout the cell cycle and at the septum during cell division (Madrid *et al.*, 2006). This pathway is partially activated by Pck2, a protein kinase C, and the small GTPase Rho2 (Barba *et al.*, 2008; Ma *et al.*, 2006). Various molecular mechanisms for CWI MAPK regulation have been reported in fission yeast, including MAPK phosphatases, mRNA stability, phosphorylation-dependent inhibition and the Sty1/Spc1 SAPK pathway (Madrid *et al.*, 2007; Sugiura *et al.*, 1998; Sugiura *et al.*, 1999; Sugiura *et al.*, 2003). The fission yeast Pck1 and Pck2 protein kinase C homologs also regulate cell wall integrity but this function may be partially independent of the CWI MAPK pathway (Barba *et al.*, 2008; Toda *et al.*, 1996).

The fission yeast *kin1* gene encodes an evolutionarily conserved serine/threonine protein kinase of the KIN1/PAR-1/MARK family (for a review, Tassan and Le Goff, 2004). Studies using disrupted *kin1::LEU2* (Levin and Bishop, 1990) or complete null *kin1*Δ (Drewes and Nurse, 2003; La Carbona *et al.*, 2004) alleles reported a role for Kin1 in maintenance of a regular rod shape. However, cells were still able to establish a longitudinal axis. A systematic characterization of the phenotypes of non-essential kinase deletion strains (Bimbo *et al.*, 2005) revealed specific stress sensitivities for the *kin1* mutant, including sensitivity to excess chloride ion and SDS. Kin1 mutant cells show an abnormally enlarged new cell end, initially referred to as the "ice-cream cone" phenotype (Levin and Bishop, 1990). In addition, Kin1 is involved in cell separation, interphase F-actin polarization and nuclear centering, and is important for completion of cytokinesis in a specific set of polarity mutants (Cadou *et al.*, 2009; La Carbona and Le Goff, 2006). The Kin1 kinase associates dynamically with the cell cortex at the cell ends during interphase in an F-actin and

microtubule independent manner. Kin1 also colocalizes with the contractile ring at mitosis and on both sides of the septum during cytokinesis (Cadou *et al.*, 2009).

Here we have investigated the role of Kin1 in the regulation of the cell surface. Kin1-GFP dynamically associates with the plasma membrane at sites of active cell wall growth. Kin1 is present in the sterol-rich fraction of membranes and its polarized localization is dependent on intact vesicular trafficking. We show that *kin1*Δ cells exhibit a thicker cell wall than wild type cells with delocalized β-glucan-containing deposits on the lateral cortex. To characterize the primary function of Kin1, we produced *kin1-as1*, an ATP analog-sensitive allele of Kin1. We show that inactivation of Kin1 triggers a rapid delocalization of plasma membrane markers of actively growing cell surface regions, including Kin1 itself. This phenotype is followed by the formation of localized Cell Wall Deposits (CWD). CWD formation is suppressed by perturbation of membrane trafficking but it is exacerbated in a *sla2* endocytic mutant. Inhibition of Kin1 also leads to the activation of the CWI MAPK module that ultimately exacerbates CWD formation. Consistent with abnormalities in the cell wall, *kin1*Δ cells are hypersensitive to various stresses. This sensitivity is alleviated by deletion of either Pck2 or any of the core CWI MAPK components. Cell Wall Deposits are partially dependent on a hyperactivated CWI MAPK pathway. We propose that the dynamic association of Kin1 with the plasma membrane is required for a robust and tight link between plasma membrane and the cell wall during vegetative growth. This function contributes to proper cell morphogenesis and tolerance to stress.

RESULTS

1) Kin1 is a plasma membrane associated protein kinase

We have previously shown that Kin1 exhibits a cell-cycle regulated and polarized localization at the cell cortex. Kin1 accumulates at the cell ends during interphase and is a component of the Contractile Actomyosin Ring (CAR) at mitosis. At septation, Kin1 is present on both sides of the septum, corresponding to new ends of the presumptive daughter cells (Cadou *et al.*, 2009).

Here, we have examined how Kin1 interacts with the cortex using sub-cellular fractionation. Integral plasma membrane GFP-Bgs4 was used as a control (Cortes *et al.*, 2002; Liu *et al.*, 2002). We observed that Kin1, like GFP-Bgs4, is present in the insoluble fraction after ultracentrifugation of a preclearing protoplast lysate obtained under non-denaturing conditions (Fig. 1A), indicating that the main pool of Kin1 is present in the membrane fraction. Next, using different extraction buffers, we observed that Kin1 is completely solubilized by 2% SDS, consistent with an interaction with membranes (Fig. 1B). Interestingly, Kin1 is not solubilized by 1% Triton X-100 at 4°C, suggesting that the major pool of Kin1 is present in the Triton X-100-insoluble lipid raft fraction (Fig. 1B). It is not solubilised by 0.5 M NaCl, 0.1 M Na_2CO_3 pH 11 or 2 M urea, so we can rule out the possibility that the major part of Kin1 is adsorbed to the plasma membrane by weak interactions (Liu *et al.*, 2002), i.e., it is not a peripheral membrane protein. A very small pool of Kin1 seems to be solubilized by 0.5 M NaCl, suggesting that different Kin1 sub-populations may be present in the plasma membrane.

These data show that Kin1 is anchored in the membrane sterol-rich fraction. Kin1 does not exhibit transmembrane domains or canonical lipid anchoring amino acid motifs (e.g., GPI anchor, CAAX box), the membrane association of Kin1 may be mediated by an as-yet-unidentified post-translational modification.

A possible link between sub-cellular fractionation (Fig. 1, A and B) and GFP-tagged localization data (Cadou *et al.*, 2009) is an association of Kin1 with sites of active membrane

flux at the plasma membrane where sterol-rich domains are polarized. To test this hypothesis, Kin1-GFP localization was assayed in cells after treatment with the ER-to-Golgi vesicular traffic inhibitor Brefeldin A (BFA, Fig. 1C). BFA treatment depolarized the sterol-rich domains detected by filipin, a histochemical stain for membrane sterols, as described by Wachtler *et al.* (2003).We observed that Kin1-GFP is delocalized over the entire plasma membrane in BFA treated cells. This suggests that Kin1-GFP polarization requires intact vesicular traffic and/or polarized sterol-rich domains.

2) Kin1 is required for proper cell wall structure

It has been shown that *kin1*Δ cells have abnormal morphologies including "ice cream cone" shaped cells and irregular cell outlines (Drewes and Nurse, 2003; La Carbona *et al.*, 2004; Levin and Bishop, 1990). In addition, *kin1*Δ cells are hypersensitive to treatment with cell wall degrading enzymes (Levin and Bishop, 1990). As shown in Fig. 2A, 77±6.5% of *kin1*Δ cells (n>400) show deposition of extra cell wall material on the cortex detected by methyl blue (beta-glucan staining), especially in the central region (hereafter collectively referred to as Cell Wall Deposits or CWD). This phenotype is exacerbated by incubating cells 5 hours at high temperature (37°C, Fig. 2A; 89.5±2.5% of cells contain CWD). Biochemical measurements consistently show a higher content of both alpha and beta glucans in the cell wall of *kin1*Δ cells compared to wild type cells (P. Perez, pers. comm.).

Because CWD in *kin1*Δ cells are predominantly located in the central region close to the septum synthesis site, we examined whether their presence might be correlated with septum synthesis. *Kin1*Δ was outcrossed to *sid4-SA1*, a thermosensitive mutant of a component of the Septation Initiation Network (SIN) that fails to septate at 37°C (Balasubramanian *et al.*, 1998). CWD were detected in the central region of *kin1*Δ *sid4-SA1* mutant cells (Fig. 2B), indicating that CWD are unlikely to correspond to excess septal

material. Similar results were observed with other SIN mutants including *cdc7-24* and *cdc11-123* (data not shown). We also detected CWD in G1-arrested *kin1*Δ *cdc10-V50* or G2-arrested *kin1*Δ *cdc25-22* cells (Fig. 2B), indicating that CWD can also form in interphase cells.

The *kin1*Δ cell wall was also examined by Transmission Electron Microscopy (TEM, Fig. 2C). This revealed a thicker cell wall and two distinct cell wall structure defects: (a) a multilayered structure on the outer face of the cell wall, and (b) the presence of cell wall depositions (called amorphous deposits), with distinct electron density between the cell wall and the plasma membrane (Fig. 2C).

Taken together, these data suggest that Kin1 deletion promotes the localized synthesis of extra cell wall material (CWD), the presence of which is probably responsible for the defects in cell morphology.

3) Using chemical genetic inhibition to study Kin1 function

To further address the primary function of Kin1 in cell wall synthesis and membrane dynamics, we used the chemical genetic approach developed by K. Shokat and colleagues (Bishop *et al.*, 2000). This strategy is based on site-directed mutagenesis of the ATP binding pocket in the catalytic domain which makes the kinase uniquely sensitive to the bulky nonhydrolyzable ATP analog 4-amino-1-*tert*-butyl-3-(1'-naphthylmethyl) pyrazolo [3,4-*d*] pyrimidine (hereafter abbreviated as 1NM-PP1). First, it was determined by comparison with other protein kinases (Bishop *et al.*, 2000) that to enlarge the ATP binding pocket of Kin1 we should introduce an F-to-G mutation at position 220 in the Kin1 Open Reading Frame (Fig. 3A). The resulting mutated kinase was named Kin1-as1 (for analog sensitive 1). Expression of Kin1-as1 from a multicopy plasmid rescued the *kin1*Δ phenotype (data not shown), indicating that Kin1-as1 is functional. Next, the wild type *kin1* ORF was replaced by a *kin1-as1* version (see Material and Methods). The resulting strain, *kin1-as1*, exhibited a wild type phenotype

indicating that the F220G mutation does not modify Kin1-as1 activity when expressed from the *kin1* locus. Furthermore, a GFP tag was inserted at the C-terminus of the Kin1-as1 ORF as described in Cadou *et al.* (2009). Expression levels of Kin1, Kin1-GFP and Kin1-as1-GFP were very similar under normal growth conditions (supplementary Fig. S1).

The kin1-as1 inhibitory phenotype was studied using increasing doses of the ATP analog 1NM-PP1. We observed that septum positions were rapidly modified with low doses of 1NM-PP1 (asterisk, Fig. 3B; supplementary Fig. S2), confirming our previous results using a repressible allele of Kin1 (Cadou *et al.*, 2009; La Carbona and Le Goff, 2006). Thus, inhibition of Kin1 kinase activity leads primarily to an off-center division site. Interestingly, we also observed the formation of localized CWD stained by methyl blue. This material was present as discrete patches in the ends of 22.2±8.9% of early G2 cells or on the lateral cortex of 10.4±3.5% of late G2 cells (arrowheads, Fig. 3B). This phenotype is reminiscent of the cell wall defects observed in *kin1*Δ cells. 1NM-PP1-mediated CWD formation was studied further in ATP analog-treated *kin1-as1* cells by TEM. We observed that CWD correspond to amorphous material deposited between the plasma membrane and the structured cell wall layers (TEM, Fig. 3B). Thus, inhibition of Kin1-as1 promoted localized accumulation of cell wall material. Furthermore, the percentage of cells containing CWD increased with increasing 1NM-PP1 concentrations (histogram, Fig. 3B).

In 1NM-PP1-containing medium, the Kin1-as1-GFP protein is dispersed in a broad region at the cell end (Fig. 3C; compare also Kin1-as1-GFP signals in DMSO or 1NM-PP1 treated cells prior to the bleaching in the FRAP experiment, Fig. 3D). Thus, the kinase activity of Kin1 is required for the maintenance of its polarized state but not for its cortical localization. Remarkably, Kin1-as1-GFP signal colocalizes with CWD (supplementary Fig. S3).

Fluorescence Recovery After Photobleaching (FRAP) allows one to monitor protein mobility in live cells (Reits and Neefjes, 2001). Here, Kin1-as1-GFP recovery was measured after laser illumination of a cell end (see Material & Methods). In the absence of 1NM-PP1, kinetics of recovery were indistinguishable between Kin1-GFP and Kin1-as1-GFP (data not shown). In cells treated with DMSO only, Kin1-as1-GFP exhibited a high mobility (the mobile fraction is ~ 60%) at the cell end (Fig. 3D). In parallel, the single type IV membrane-spanning protein GFP-Psy1 was almost immobile under identical FRAP conditions. Interestingly, in cells treated with 1NM-PP1, Kin1-as1-GFP protein showed a marked reduction in mobility. This suggests that the kinase activity of Kin1contributes to its mobility.

4) Kin1 is required for plasma membrane organization

Cell wall structure dynamics depend on the polarization of the growth machinery at the cell ends and at the division site (septum). Active cell wall synthesis sites are intimately linked with polarized F-actin structures, trafficking and sterol-rich plasma membrane domains (Wachtler *et al.*, 2003). We previously showed that *kin1*Δ cells exhibit a depolarized interphase F-actin cytoskeleton on the lateral cortex (La Carbona and Le Goff, 2006). Here, we have monitored localization of the sterol-rich domains in the plasma membrane using the fluorescent dye filipin. In contrast to wild-type cells, *kin1*Δ cells show depolarized sterol-rich domains on the entire plasma membrane (Fig. 4A).

TEM studies did not reveal accumulation of vesicles in the cytoplasm (Fig. 2C), suggesting that general secretion might not be defective in *kin1*Δ cells. We therefore examined the localization of the fission yeast t-SNARE syntaxin homolog GFP-Psy1, a component of the late secretion SNARE complex. In *kin1*Δ cells, GFP-Psy1 was detected on the overall plasma membrane, as in wild type cells, suggesting that Kin1 does not regulate GFP-Psy1 plasma membrane targeting (Fig. 4B). However, we observed that GFP-Psy1

accumulated on the lateral cortex (arrowheads, Fig. 4B), indicating that Kin1 affects the distribution of GFP-Psy1 within the plasma membrane.

Polarization of plasma membrane sterol-rich domains (and cell wall synthesis) is dependent on efficient intracellular trafficking. In yeast, the amphiphilic fluorescent dye FM4-64 is taken up by endocytosis and transported to the vacuolar membrane. When living *kin1*Δ cells incorporate the FM4-64 dye (Fig. 4C), stained vacuoles exhibit morphology defects. Vacuoles seemed strikingly smaller and more numerous in *kin1*Δ cells compared to wild type cells, suggesting a defect in vacuole biogenesis. Time-lapse studies revealed that initial incorporation of FM4-64 was not affected in *kin1*Δ cells but that later the signal of FM4-64 was more diffuse than in wild type cells (supplementary Fig. S4).

During vegetative growth, polarized cell wall synthesis is dependent on the localization of transmembrane beta-glucan synthase holoenzymes at cell wall remodeling sites (Cortes *et al.*, 2002; Cortes *et al.*, 2005; Cortes *et al.*, 2007; Liu *et al.*, 2002; Martin *et al.*, 2003). We therefore monitored the localization of GFP-tagged vegetative beta-glucan synthase catalytic subunits Bgs1, Bgs3 and Bgs4. *Kin1*Δ cells were not defective in polarized localization of GFP-Bgs3 (supplementary Fig. S5). By contrast, GFP-Bgs1 and GFP-Bgs4 signals were not only observed at cell wall remodeling sites but could also be detected on the lateral cortex of *kin1*Δ cells (Fig. 4D; supplementary Fig. S5). In addition, Western blot analysis revealed that expression of Bgs1 and Bgs4, but not Bgs3, was significantly upregulated in the absence of Kin1 (supplementary Fig. S6). Thus, Kin1 regulates Bgs1 and Bgs4 expression as well as their polarization at the plasma membrane.

If in the *kin1*Δ mutant Bgs1 is more stable at the plasma membrane, the *kin1*Δ mutation should lower the sensitivity to temperature of *cps1-191,* a thermosensitive allele of *bgs1*. Indeed, we observed growth rescue at 32°C and 37°C in the *kin1*Δ *cps1-191* double mutant compared to the single *cps1-191* mutant (Fig. 4E).

To characterize the primary function of Kin1 at the plasma membrane, we used the rapid inactivation of Kin1-as1 mediated by 1NM-PP1. We observed that sterol-rich domains are depolarized when 1NM-PP1 is added to the culture medium (Fig. 5A). Since overall endocytosis is not affected in the *kin1*Δ mutant, one explanation for the increased stability of the beta-glucan synthase Bgs1 at the plasma membrane is that a specific regulator of the endocytosis of Bgs1 is altered. Cfh3 is a protein that colocalizes and co-immunoprecipitates with Bgs1 and regulates its stability at the plasma membrane (Sharifmoghadam and Valdivieso, 2009). To test if the early phenotype of Kin1 inactivation affects Cfh3 regulation, we followed GFP-Cfh3 in a *kin1-as1* background. We observed that the cell-cycle regulated distribution of GFP-Cfh3 was perturbed in 1NM-PP1-treated cells. In particular, GFP-Cfh3 was strikingly depolarized at the plasma membrane compared to control cells (Fig. 5B). Delocalized Cfh3-GFP coincided with sites of CWD formation (supplementary Fig. S3). Moreover, 1NM-PP1-treatment of *GFP-Psy1 kin1-as1* cells also showed that GFP-Psy1 localization was perturbed and that GFP-Psy1 accumulates at sites of CWD formation (supplementary Fig. S3). These observations link the role of Kin1 in proper localization of GFP-Cfh3 and GFP-Psy1 with cell wall regulation.

Next, we determined the kinetics of GFP-Cfh3 depolarization and CWD formation by analyzing samples in the fluorescence microscope during a time-course experiment following inhibition of Kin1-as1 with 1NM-PP1 (Fig. 5C). The results show clearly that depolarization of GFP-Cfh3 is nearly complete after 60 minutes and precedes the accumulation of cell wall material in CWD. This demonstrates that the primary function of Kin1 resides in plasma membrane organization and suggests that CWD is a consequence of the disruption of this primary function. In parallel, activation of the Cell Wall Integrity Pmk1 MAP kinase was determined. Pmk1 basal phosphorylation was detected at different time points by employing an anti-phospho p42/p44 antibody as described earlier (Madrid *et al.*, 2006). Pmk1 activation

was only obvious after 90 minutes (Fig. 5D), supporting the idea that this phenotype may be a consequence of CWD formation.

In summary, our data suggest that Kin1 function is primarily required for proper polarization of sterol-rich domains and proper localization of regulators of cell wall synthesis at the plasma membrane. They suggest further that the initial effect of inhibition of Kin1 is depolarization of the cell wall synthesis machinery, which then leads to CWD. Presence of CWD then triggers the CWI pathway which further exacerbates the formation of CWD.

5) A possible role for Kin1 in maintenance of a robust link between plasma membrane and the cell wall during vegetative growth

A possible explanation for the generation of CWD could be a localized invagination of the plasma membrane due to a modification of the mechanical properties of the lipid bilayer. This could result from a loss of a robust and tight link between the cell wall and the plasma membrane at the cell surface. The enlarged periplasmic space could then be filled by amorphous cell wall material (Figs. 2 and 3). How could the plasma membrane invaginate into the cytoplasm? One possibility is that a defect in the balance between exocytic and endocytic vesicles plays a role.

It is unlikely that Kin1 function regulates exocytic vesicle fusion because no accumulation of large vesicles is observed by TEM in *kin1*Δ cells or during Kin1-as1 inhibition. Following our hypothesis, we reasoned that inhibition of active vesicular trafficking would suppress CWD formation since cell wall material is targeted to the cell cortex by secretion machinery. Indeed, *kin1-as1* cells pre-treated with BFA, an inhibitor of ER to Golgi transport, showed a complete suppression of CWD formation after 1NM-PP1 addition (Fig. 6A). By comparison, CWD were observed in 39.2% of control cells. However, cells treated with both BFA and 1NM-PP1 still showed asymmetric septa, demonstrating that

1NM-PP1 is able to inactivate Kin1-as1 in the presence of BFA and strongly suggesting that nuclear mispositioning and CWD formation are distinct phenotypes. Consistently, no CWD were observed in an ATP analog-treated *kin1-as1 for3*Δ mutant, where no interphase F-actin cables are nucleated (data not shown). Similarly, a strong reduction of CWD (6.4% compared to 45.7% in a *myo4*$^{+}$ background) was observed in a 1NM-PP1-treated *kin1-as1 myo4*Δ mutant, where exocytic vesicle transport was disturbed (Fig. 6B). A corollary to our hypothesis is that an inhibition of the endocytosis process should stimulate CWD formation. To test this, we used the *sla2*Δ strain which is deleted for the End4/Sla2 transmembrane protein. Interestingly, control *sla2*Δ cells stained with methyl blue dye showed discrete punctate staining on the lateral cortex (Fig. 6B). This indicates that the *sla2*Δ mutation itself promotes formation of localized CWD, consistent with an imbalance between endocytosis and exocytosis. Moreover, when Kin1-as1 was inhibited by 1NM-PP1, a dramatic increase in both the number and intensity of CWD was observed (Fig. 6B). Thus, the endocytosis defect exacerbates kin1-as1-mediated CWD formation.

Next, we monitored Kin1-GFP localization in the mutants *for3*Δ, *myo4*Δ and *sla2*Δ (data not shown). Kin1-GFP was not strictly accumulated at cell ends in any of these situations. We conclude that vesicular trafficking defects (either exocytosis or endocytosis) promote depolarization of Kin1-GFP around the cell cortex, even though Kin1-GFP remains at the plasma membrane. We also monitored Kin1-GFP mobility by FRAP in *for3*Δ, *myo4*Δ, and *sla2*Δ mutants and in BFA-treated cells. Only BFA alters Kin1-GFP mobility and that only moderately (Supplementary Fig. S7). Since BFA is a strong inhibitor of general membrane trafficking and polarization of sterol-rich domains, this observation suggests that the altered mobility of Kin1-GFP in this situation may be due to broad BFA-mediated perturbation of plasma membrane organization.

6) The Pmk1 MAP kinase pathway affects CWD formation in *kin1*Δ cells.

In yeast cells, cell wall integrity (CWI) MAPK module is involved in the sensing of and response to perturbations in the cell wall and the plasma membrane (Levin, 2005; Barba *et al.*, 2008). In *S. pombe* the cell integrity MAP kinase module is composed of MAPKKK Mkh1, MAPKK Pek1 and MAPK Pmk1, the key element of the pathway. The protein kinase C ortholog Pck2 and the small Rho GTPase Rho2 act upstream of the Mkh1-Pek1-Pmk1 cascade (Ma *et al.*, 2006; Barba *et al.*, 2008), and they are responsible for Pmk1 activation in response to several stresses such as hypertonic or hypotonic shock (Barba *et al.*, 2008). The presence of altered cell walls in the *kin1*Δ mutant suggested that Pmk1 activity might be deregulated in these cells. CWD formation was monitored in a *kin1*Δ *pmk1*Δ double mutant. As shown in Table 2 and supplementary Fig. S8, deletion of *pmk1*$^+$ reduced the number of CWD in *kin1*Δ cells. Identical results were obtained with deletions of *mkh1*$^+$ and *pek1*$^+$ (data not shown). Similarly, CWD formation was partially abolished in *kin1-as1* cells disrupted in either *pmk1*$^+$, *pek1*$^+$ or *mkh1*$^+$, and treated with 1NM-PP1 (data not shown). Partial suppression of CWD was also observed in *kin1*Δ *pck2*Δ cells, indicating that Pck2 is also involved in the formation of CWD (Table 2; supplementary Fig. S8). The fact that *pck2*Δ suppresses CWD more strongly than *pmk1*Δ suggests that Pck2 is also involved in CWD formation independently of the Pmk1 pathway. This result is not surprising since Pck2 regulates cell wall integrity independently of the CWI cascade (Toda *et al.*, 1996; Barba *et al.*, 2008). Moreover, CWD in *kin1*Δ cells were not markedly affected by deletion of *rho2*$^+$ (Table 2; supplementary Fig. S8), suggesting that the Pmk1 pathway may branch at the Rho2 level. On the other hand, Pmk1 hyperactivation elicited by deletion of the dual specificity phosphatase *pmp1*$^+$ (Sugiura *et al.*, 1998) significantly increased the number of CWD in *kin1*Δ cells (Table 2; supplementary Fig. S8).

We also tested the possible role of Pck1, a Pck2 homolog, in CWD formation. Pck1 negatively regulates cell wall integrity and the Pmk1 pathway, but its function remains obscure (Arellano *et al.*, 1999; Barba *et al.*, 2008). A *kin1*Δ *pck1*Δ double mutant showed extensive CWD (Table 2; supplementary Fig. S8) and severe growth defects, supporting the existence of synthetic interaction between the single mutants.

The above results suggested the existence of a specific link between Pmk1 activity and CWD formation in *kin1*Δ cells. To further explore this hypothesis, we first determined Pmk1 basal phosphorylation in growing cells from the above mutants by employing the anti-phospho p42/44 antibody. As can be seen in Fig. 7A, Pmk1 was hyperphosphorylated in the *kin1*Δ mutant. Importantly, the increase in Pmk1 phosphorylation elicited by *kin1*$^+$ deletion was abolished in *kin1*Δ *pck2*Δ cells but not in the *kin1*Δ *rho2*Δ mutant (Fig. 7A). Moreover, basal Pmk1 phosphorylation was higher in either *kin1*Δ *pmp1*Δ or *kin1*Δ *pck1*Δ cells than in their respective single mutant counterparts (Fig. 7A).

In *S. pombe,* calcineurin and Pmk1 play antagonistic roles in chloride homeostasis, and Pmk1 hyperactivation leads to strong sensitivity to this anion (Sugiura *et al*., 1998). Pmk1 hyperphosphorylated cells lacking *kin1*$^+$ showed an evident growth inhibition in YES medium supplemented with 0.2 M $MgCl_2$ (Fig. 7B), and this phenotype was rescued by additional deletion of *pmk1*$^+$ or *pck2*$^+$genes (Fig. 7B). Interestingly, disruption of either *pck1*$^+$ or *pmp1*$^+$ in a *kin1*Δ background clearly increased cell sensitivity to chloride anions as compared to single *kin1*Δ , *pck1*Δ or *pmp1*Δ parental strains (Fig. 7B).

As a whole, our results strongly suggest that in fission yeast: (i) Kin1 operates at the plasma membrane and its function influences Pmk1 activity *via* Pck2 but in a Rho2-independent fashion; (ii) hyperactivation of the Pmk1 MAPK pathway is partially responsible for the production of CWD in the absence of Kin1 kinase; and (iii) other Pmk1-independent mechanisms are also important in CWD formation.

The cell wall is essential for determination of cell shape and also for mechanoresistance to environmental changes. Since lack of Kin1 function alters cell wall organization, *kin1*Δ cells might behave differently from wild type cells under stress conditions.

Congruent with a strong cell separation defect at high temperature (Levin and Bishop, 1990), *kin1*Δ cells show reduced growth at 37°C (data not shown). We monitored the effect of plasma membrane stress and cell wall damaging agents on *kin1*Δ cell growth to show that these cells are extremely sensitive to stress induced by low doses of SDS and to the cell wall damaging agent Calcofluor white (Fig. 7C), but not to the beta-glucan synthase inhibitor caspofungin (data not shown). Thus, our data indicate that Kin1 function is required for cell tolerance to specific stress conditions, and confirm those obtained by Bimbo *et al.* (2005) during a systematic study of non-essential kinase deletions. Importantly, deletion of $pmk1^{+}$ or $pck2^{+}$ alleviated the hypersensitivity of *kin1*Δ cells to either SDS or Calcofluor white (Fig. 7C; data not shown). Thus, our results indicate that Kin1 function is required for resistance to thermal and cell wall stresses, and that Pmk1 activity is involved in this response.

7) SICS and CWD

Recently, Robertson and Hagan have described the formation of Stress-Induced Calcofluor Structures (SICS) upon treatment of fission yeast cells with either 1.2 M Sorbitol or 0.6 M KCl (Robertson and Hagan, 2008). During the course of this study, we have observed that treatment of cells with 0.01% SDS can also promote SICS (supplementary Fig. S9).

SICS appear to be very similar to the CWD described in the present work. We therefore examined whether Kin1 is required for SICS formation. Incubation in Sorbitol or SDS exacerbated cell wall deposits in *kin1*Δ cells (supplementary Fig. S9). In addition, *kin1-as1* cells treated with both 1NM-PP1 and Sorbitol or SDS showed additive effects on

accumulation of cell wall material compared to 1NM-PP1-treated control cells (data not shown). These observations strongly suggest that Kin1 is dispensable for SICS formation.

DISCUSSION

Proper regulation of cell morphogenesis is essential for eukaryotic cells to carry out their functions, divide and adapt to environmental changes. Here, we have demonstrated a role for the fission yeast Kin1 kinase in controlling cell surface organization.

Kin1 associates dynamically with the plasma membrane

Fission yeast Kin1 is structurally related to the evolutionary conserved PAR-1/MARK protein kinase family (Tassan and Le Goff, 2004). These are plasma membrane proteins, as demonstrated by sub-cellular fractionation experiments in budding yeast and plasma membrane localization in metazoan systems (Chartrain *et al.*, 2006; Elbert *et al.*, 2005; Hurov *et al.*, 2004; Tibbetts *et al.*, 1994; Vaccari *et al.*, 2005). Fission yeast Kin1-GFP is detected at the plasma membrane and accumulates in actively growing cell surface regions. Kin1-GFP signal does not overlap exactly with methyl blue. Kin1 is probably present on the cytoplasmic face of the plasma membrane and thus in a slightly different position from the cell wall. A similar conclusion has been reached for the budding yeast homologs Kin1p and Kin2p (Tibbetts *et al.*, 1994). Moreover, our results consistently show that Kin1 associates with the Triton X-100-insoluble membrane fraction, suggesting that it is preferentially located in sterol-rich lipid rafts. However, *in silico* analysis of the Kin1 ORF does not reveal any known membrane association domains such as FYVE, PH, CAAX box, GPI anchoring motifs or transmembrane domains. Moreover, it is not detached from the membrane by 0.5M NaCl or 2M urea, indicating that this is not simply a peripheral membrane protein. This suggests that

Kin1 may be anchored in the lipid membrane by an as-yet-unidentified post-translational modification.

Our FRAP studies reveal that Kin1 interaction with the plasma membrane is highly dynamic compared to a membrane spanning protein. This high mobility is evidently not influenced by plasma membrane viscosity, but we show that it does require Kin1 kinase activity, suggesting a dynamic interaction with a putative membrane protein substrate. This is a puzzling observation considering that Kin1 associates with the membrane fraction. An alternative possibility is that inhibition of Kin1-as1 rapidly alters plasma membrane properties and this may eventually modify Kin1 dynamics. It will be necessary to decipher the underlying molecular mechanism regulating the association of Kin1 with the membrane to address this issue.

A role for Kin1 in regulating the cell surface

Budding yeast Kin1 homologs, Kin1p and Kin2p, have been isolated as multicopy suppressors of several mutants acting at different levels of the secretory pathway, including the *cdc42-6* and *rho3-V51* alleles. Kin1p and Kin2p have been shown to interact with and regulate phosphorylation of proteins acting at the final stage of exocytosis such as the t-SNARE Sec9p. Epistatic data suggest a role for Kin1p and Kin2p downstream of *CDC42* and *RHO3* and upstream of vesicle fusion (Elbert *et al.*, 2005).

In contrast to budding yeast, *sec9* is essential in fission yeast (Nakamura *et al.*, 2005) and vesicular trafficking mutants in fission yeast remain poorly characterized (Takegawa *et al.*, 2003). Exocytic mutants such as *rho3*Δ or the exocyst component *sec8-1* have been described which accumulate vesicles about 100 nm in diameter, indicating a defect in vesicle fusion with the plasma membrane (Wang *et al.*, 2002; Wang *et al.*, 2003). However, we did

not detect such vesicles in *kin1*Δ by TEM. In addition, we were unable to detect suppression of these mutants by Kin1 overexpression in fission yeast (data not shown). Growth of *kin1*Δ *rho3*Δ cells is severely reduced at 25°C and inhibited above that temperature. Electron microscopy studies show that *kin1*Δ *rho3*Δ cells exhibit tremendous cell wall accumulation at 36°C (unpublished results), suggesting that Rho3 and Kin1 act in nonredundant pathways to regulate the cell surface. Thus, budding and fission yeast Kin1 homologs may control a late step in plasma membrane organization, such as membrane recycling, even though molecular mechanisms may differ. In higher eukaryotes, mammalian Par-1 may regulate exocytosis although Par-1 does not interact with the Sec9 SNAP25 homolog (cited in Elbert *et al.*, 2005).

The early defect of Kin1 loss-of-function impinges on several aspects of plasma membrane organization such as localization of t-SNARE and beta-glucan synthases and polarization of sterol-rich domains. With regard to Kin1 localization, our data are consistent with a primary role for Kin1 in maintenance of polarized actively growing cell surface regions in the plasma membrane. We propose that Kin1 deletion or inhibition may promote localized loss of cohesiveness between the plasma membrane and the cell wall. Exocytic and cell-wall regulators are concentrated at these sites and thus the periplasmic space formed by this loss of cohesiveness would be rapidly filled up by amorphous cell wall deposits. A possible role for phosphorylation catalyzed by the Kin1 kinase might therefore be the recycling of proteins transiently present at the plasma membrane, including Kin1 itself and cell wall synthesizing enzymes. In conclusion, Kin1 function would be required to ensure a robust and tight link between the plasma membrane and the cell wall during vegetative growth.

Kin1 inhibition causes plasma membrane depolarization which leads to cell wall damage and hyperactivation of the CWI Pmk1 pathway

Cell wall damage and plasma membrane stretch are detected and relayed by transmembrane protein sensors connected with the cell wall via post-translational modifications. Following detection, the CWI MAPK pathway plays a major role in intracellular signalling to promote an adapted response (Barba *et al.*, 2008; Levin, 2005). We propose that *kin1* deletion or inhibition mimics continuous cell wall or plasma membrane stress. In *kin1*Δ cells, the beta-glucan synthase Bgs1 is more stable at the plasma membrane than in wild type cells. Cfh3 is a specific regulator of Bgs1 endocytosis. Time-course experiments consistently show that the primary effect of Kin1-as1 inhibition is the rapid depolarization of Cfh3 and this may impair Bgs1 endocytosis. Accumulation of Cell Wall Deposits (CWD) is not detected until later and Pmk1 activation is detected even later, suggesting that CWI pathway is activated as a consequence of the plasma membrane and cell wall defects. Production of empty periplasmic spaces as a result of Kin1 inhibition may lead to a cellular response involving a rapid compensation by synthesis of amorphous cell wall material. Kin1 inhibition may also trigger (either directly or indirectly *via* induction of CWD) a permanent signal of cell surface injury, activating the CWI pathway and leading to more CWD than would result from Kin1 inhibition alone. Inhibition of CWI signalling suppresses CWD formation, indicating that CWI contributes to such a cell wall response. However, this suppression is only partial, suggesting that other molecular pathways are also involved. Sty1 MAPK, the core component of the Stress-Activated Protein Kinase (SAPK) pathway in fission yeast, may also participate in CWD formation. However, *kin1*Δ *sty1*Δ cells did not show any significant change in the number of CWD compared to *kin1*Δ alone (data not shown).

Kin1, Cell Wall Integrity and Sensitivity to Stress

The presence of CWD may be detrimental to the cell's capacity to resist specific stresses, specifically those which challenge cell wall plasticity. Thus, *kin1*Δ cells are hypersensitive to

several stress conditions, including upward shifts in osmotic pressure and temperature. *Kin1*Δ cells have a higher content of beta- and alpha-glucan (P. Perez, pers. com.) and we have shown that the expression of Bgs1 and Bgs4 is upregulated in these cells. This is consistent with *kin1*Δ cells being insensitive to caspofungin, an inhibitor of cell-wall synthesizing enzymes. Inhibition of the CWI pathway reduces cell wall thickening and contributes to the alleviation of stress sensitivity.

The function of fission yeast Pck1 is largely unknown, but its physical interaction with Rho GTPases and genetic interactions with *pck2*Δ and other cell wall regulating genes suggest a function in cell wall integrity (Arellano *et al.*, 1999; Calonge *et al.*, 2000; Ma *et al.*, 2006; Toda *et al.*, 1996). Our work reveals a strong synthetic interaction between *kin1*Δ and *pck1*Δ. In double mutant cells, growth and cell polarity are severely compromised. We conclude that Kin1 is required for the maintenance of cell integrity in the absence of Pck1 whereas the *kin1*Δ *pck2*Δ mutant shows phenotypic suppression. Thus, the *kin1*Δ mutation could be useful in discriminating the distinct functions of these protein kinase C homologs.

SICS versus CWD: an identical consequence of two unrelated causes?

It is tempting to speculate that SICS and CWD are the same consequence of plasma membrane disruption, occuring when wild type cells are subjected to stress or when Kin1 is inhibited under normal growth conditions, respectively. For example, we have suggested that formation of CWD in the absence of Kin1 activity may be due to loss of cohesiveness between the cell wall and plasma membrane and subsequent accumulation of amorphous cell wall material. A similar loss of cohesiveness could result from hypertonic treatment (in the case of 1.2 M sorbitol or 0.6 M KCl) or damage to the plasma membrane (in the case of SDS). However, SICS are not dependent on the CWI pathway, whereas CWD are partially

dependent on that pathway. This suggests that SICS and CWD may correspond to different cellular defects that only appear to have similar outcomes.

Kin1 cooperates with Sla2-dependent membrane internalization and acts downstream of Myo4 and For3

A mutation like *sla2*Δ that alters endocytosis promotes accumulation of cell wall material at the plasma membrane (this study; Ge *et al.*, 2005), perhaps due to inhibition of membrane internalization. Fluorescent FM4-64 uptake suggests that Kin1 has no significant role in endocytosis itself but inhibition of endocytosis *via* the *sla2*Δ mutation exacerbates *kin1-as1*-mediated CWD formation. This and other observations support the notion that inability of cells to properly regulate membrane internalization promotes the accumulation of cell wall material between a layered cell wall and the plasma membrane.

Vesicles are transported *via* F-actin cables to sites of polarized growth by molecular motors such as the type V myosin Myo4 (Feierbach and Chang, 2001; Motegi *et al.*, 2001; Mulvihill *et al.*, 2006; Win *et al.*, 2001). In accordance with a Kin1 function in membrane recycling at the cell cortex, we show that inhibition of general vesicle targeting to the plasma membrane by deletion of *myo4*, by BFA-mediated interruption of intracellular trafficking or by inhibition of the formation of F-actin cables through *for3* deletion in the *kin1-as1* background, suppresses the CWD phenotype usually associated with Kin1 inhibition. Myo4 has been shown to target the beta-glucan synthase Bgs1 protein to sites of polarized growth (Mulvihill *et al.*, 2006). Here we show that Kin1 is not involved in targeting Bgs1, nor its regulator Cfh3, to the plasma membrane but in their restriction to polarized growth sites. Rather than regulating exocytic and/or endocytic flux *per se*, Kin1 appears to regulate the balance between these processes, both spatially and dynamically.

EXPERIMENTAL PROCEDURES

S. pombe strains, media and reagents

S. pombe strains used in this study and their genotypes are listed in Table 1. Media (EMM and YES) and genetic methods were as described (Moreno *et al.*, 1991). Cells were grown at 25°C, 30°C or 37°C for 5h when stated. Genetic crosses and sporulation were performed in EMM agar plates with 1/10 limiting nitrogen source NH_4Cl. Tetrad dissection was performed with a Singer MSM system (Somerset, UK) and genotypes of interest were selected by appropriate replica plating. GFP-Psy1-expressing strains were derived from *leu1-32 h-* (WT) or *kin1::kanMX6 leu1-32* h- (*kin1*Δ) cells transformed with the pTN381 (Psy1 promoter-GFP-Psy1) plasmid (a kind gift from T. Nakamura, Osaka, Japan) integrated at the *leu1* locus. For nonstandard growth conditions, cells were grown in liquid media containing 0.01% Sodium-Dodecyl-Sulfate (SDS), 1.2 M Sorbitol, or 50 μg/ml Brefeldin A (diluted in ethanol, Sigma). For stress induction conditions, EMM- or YES-based agar plates contained 0.1M or 0.2M $MgCl_2$, 0.005% SDS, or 0.5 mg/ml Calcofluor white. Exponentially growing cells of different strains were diluted and 10^5, 10^4,10^3, 10^2 cells were spotted for 3-5 days at 30°C. The ATP-analog 4-Amino-1-*tert*-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (Bishop et al., 2000), abbreviated as 1NM-PP1, was synthesized as described in Dischinger *et al.* (2008), prepared as a 25 mM stock solution in DMSO and stored at -20°C.

Mutagenesis and integration of *kin1-as1*

Mutagenesis of phenylalanine to glycine at position 220 in the Kin1 ORF was performed using a "QuickChange Multi Site-Directed Mutagenesis" kit (Stratagene) and the pREP41GFP-Kin1 plasmid as a DNA matrix (La Carbona *et al.*, 2004). The resulting pREP41GFP-kin1-as1 (F220G) plasmid was fully sequenced and transformed into

kin1::kanMX6 leu1-32 (*kin1*Δ) cells. Expression of the mutated Kin1-as1 protein fully rescued all tested *kin1*Δ phenotypes (cell morphology, zymolyase and salt resistance, septation efficiency), indicating that the mutated Kin1-as1 protein was functional (data not shown). The mutagenized ORF was amplified by PCR and transformed into *kin1::LEU2* disrupted cells (Levin and Bishop, 1990). Kin1-as1 integrants were selected by growth on EMM agar plates containing 1 M KCl that did not allow *kin1*Δ growth. Leucine auxotrophy due to loss of the *LEU2* marker confirmed integration at the *kin1* locus. The kin1 ORF was further modified by homologous recombination using the Gly5-GFP-kanMX cassette as described (Bahler *et al.*, 1998; Cadou *et al.*, 2009). G418-resistant colonies were selected and the *kin1-as1-GFP* ORF was fully sequenced on the genome. Inhibition of Kin1-as1 was performed using 1NM-PP1 diluted from a 25 mM stock solution in DMSO. Except when otherwise stated, cells were incubated 2h with 20 μM 1NM-PP1 at 30°C.

Western blotting

A 50 ml cell culture was harvested by centrifugation at 3,000 rpm, washed in 20 ml ice-cold PBS and then in 10 ml ice-cold STOP buffer (10 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.05% NaN_3). Dry cell pellets were stored at -70°C. Lysis was carried out by vortexing cells in 200 μl of lysis buffer (10% glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% nonidet P-40, 15 mM imidazole, Roche protease inhibitor cocktail) supplemented with 2% SDS and 0.3 g of glass beads (Sigma). The cell lysate was then centrifuged for 10 min. at 13,000 rpm at 4°C and the supernatant was diluted with 3X Laemmli loading buffer. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose or PVDF Immobilon-P membranes. Membranes were blotted in PBS/5% milk/0.1% Tween 20. Rabbit polyclonal anti-Kin1 (La Carbona *et al.*, 2004) and mouse monoclonal anti-GFP (Roche) were used at 1:3000 and 1:1000 dilutions, respectively. For a loading control, mouse monoclonal anti-PSTAIR (anti-

Cdc2, Sigma) was used at 1:5000 dilution. Secondary antibodies were conjugated to either alkaline phosphatase or horseradish peroxidase and revealed by ECF/Storm or West DURA (Pierce), respectively. Activated Pmk1 was detected in total cell extracts using a rabbit anti-p42/44 antiphospho-antibody at 1:1000 dilution and the ECL system as described (Madrid *et al.*, 2006).

Sub-cellular fractionation

Sub-cellular fractionation was performed using 200 ml of exponentially growing cells. Cells were incubated for 10 min. in RB buffer (1.2 M Sorbitol, 30 mM beta-mercaptoethanol, 50 mM Tris pH 8.5) and washed twice in SP buffer (1.2 M Sorbitol, 50 mM NaH_2PO_4 pH 5.8). Cells were then incubated 1h45 at 30°C in SP buffer containing 10 mg/ml Lysing enzymes (Sigma). The resulting spheroplasts were checked by phase contrast microscopy and spun at 3,000 rpm for 5 min. at 4°C. Spheroplasts were resuspended in 250 µl of L buffer (365 mM sucrose, 20 mM MOPS pH7.4, Roche protease inhibitor cocktail) and lysed under nondenaturing conditions using a Dounce homogenizer until 90% lysis efficiency was achieved. Cellular debris was removed by centrifugation at 3,000 rpm for 5 min. at 4°C. The cell lysate was then spun at 100,000 g for 1h at 4°C and the pellet and supernatant corresponded to the membrane and cytoplasmic fractions, respectively. The membrane fraction was resuspended in TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, Roche protease inhibitor cocktail) and different aliquots were pelleted at 100,000 g for 30 min. at 4°C and resuspended in TNE buffer, TNE+0.5 M NaCl, TNE+0.1 M Na_2CO_3 pH11, TNE+2 M urea, TNE+1% Triton X-100, and TNE+2% SDS. After 30 min. on a rotating wheel and further centrifugation at 100,000 g for 30 min. at 4°C, supernatants and pellets were analyzed by Western blot.

Microscopy techniques

Cells were observed after reaching the exponential growth phase. For time-lapse video microscopy, 2 µl of cells were mounted on 2% EMM agarose pads. GFP movies were captured using a spinning disk Nikon TE2000 microscope with a 100x 1.45 NA PlanApo and a HQ2 Roper camera. Incorporation of FM4-64 (Molecular Probes) was recorded in live cells (1 image per 10 min) on agarose pads. Sterol-rich domains were detected using 5 µg/ml filipin (Sigma) and a DMRXA Leica microscope equipped with a neutral filter. For cell wall and septum detection, cells were fixed with 4% formaldehyde (Sigma) for 30 min., washed in PBS and stained with 0.5 mg/ml methyl blue (Sigma). Cells were observed using a DMRXA Leica microscope with a 100x 1.45 NA PlanApo and a CoolSNAP ES camera. Cell lengths were measured by ImageJ software. Colocalization experiments between cell wall (stained by methyl blue) and GFP tagged membrane proteins and Fluorescence Recovery After Photobleaching (FRAP) were performed on a spinning disc Nikon TE2000 microscope and used the Metamorph software. For FRAP, GFP signals were recorded every second and the bleach was applied for 0.5 sec. Cells were then observed every second for 2 min. Normalized FRAP data were calculated by the ImageJ software. For Transmission Electron Microscopy, cells were stained with potassium permanganate. Images were captured by a Jeol Jem-1010 (Peabody, MA).

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3

Table 1: *S. pombe* strains used in this study.

strain	Genotype	Reference
XLG009	*h- cdc10-V50 leu1-32*	(Reymond *et al.*, 1992)
XLG029	*h- leu1-32*	Lab stock
XLG031	*h- cdc25-22 leu1-32 ura4-D18*	(Russel and Nurse, 1986)
XLG046	*h- kin1::kanR cdc25-22 leu1-32*	(La Carbona and Le Goff, 2006)
XLG053	*h- leu1-32 kin1::kanMX6*	(La Carbona *et al.*, 2004)
XLG092	*h- kin1::kanR cdc10V50 leu1-32*	This study
FM401	*h- myo4::ura4+ ade6-M216 leu1-32 ura4-D18*	(Motegi *et al.*, 2001)
PPG3847	*h- cps1-191 ura4-D18*	(Liu *et al.*, 1999)
PPG165	*h- pck1::ura4+ leu1-32 ura4-D18*	(Arellano *et al.*, 1999)
PPG166	*h- pck2::LEU2 leu1-32*	(Arellano *et al.*, 1999)
XLG431	*h+ kin1::kanR pck1::ura4+ leu1-32 ura4-D18*	This study
XLG454	*h- kin1::kanR pck2::kanR leu1-32 ura4-D18*	This study
XLG485	*h+ kin1::kanR pmk1::ura4+ leu1-32 ura4-D18*	This study
XLG510	*h- sid4-SA1 leu1-32*	(Balasubramanian *et al.*, 1998)
XLG520	*h+ kin1::kanR sid4-SA1*	This study
#519	*h- his3-D1 ura4-D18 leu1-32 bgs1::ura4*$^+$ P_{bgs1+}*::GFP-bgs1*$^+$*:leu1*$^+$	(Cortes *et al.*, 2005)
#1216	*h- his3-D1 ura4-D18 leu1-32 bgs3::ura4*$^+$ P_{bgs3+}*::GFP-bgs3*$^+$*:leu1*$^+$	(Cortes *et al.*, 2005)
#561	*h- his3-D1 ura4-D18 leu1-32 bgs4::ura4+ Pbgs4+::GFP-bgs4+:leu1+*	(Cortes *et al.*, 2005)
XLG535	*h- kin1::kanR bgs1::ura4*$^+$ P_{bgs1+}*::GFP-bgs1*$^+$*:leu1*$^+$ *leu1-32 ura4-D18*	This study
XLG536	*h- kin1::kanR bgs3::ura4*$^+$ P_{bgs3+}*::GFP-bgs3*$^+$*:leu1*$^+$ *leu1-32 ura4-D18*	This study
XLG537	*h- kin1::kanR bgs4::ura4*$^+$ P_{bgs4+}*::GFP-bgs4*$^+$*:leu1*$^+$ *leu1-32 ura4-D18*	This study
TP319-13C	*h- pmk1::ura4+ ura4-D18*	(Toda *et al.*, 1996)
XLG572	*h- kin1::Gly5-GFP-kanR leu1-32 ura4-D18*	(Cadou *et al.*, 2009)
XLG595	*h- kin1-as1::Gly5-GFP-kanR ade6-704 leu1-32 ura4-294*	This study
XLG602	*h+ rho2::kanR ade6- ura4-D18 leu1-32*	(Ma *et al.*, 2006)
XLG605	*h+ pmp1::kanR ade6- leu1-32 ura4-D18*	(Sugiura *et al.*, 1998)
XLG614	*h- kin1::kanR rho2::kanR leu1-32 ura4-D18*	This study
XLG615	*h- pmp1::kanR kin1::kanR ade6- leu1-32*	This study
XLG620	*h+ kin1-as1::Gly5-GFP-kanR sla2::kanR ade6- leu1-32 ura4-*	This study
XLG679	*h- leu1:*P_{psy1}*- GFP-psy1*	(Nakamura *et al.*, 2001)
XLG680	*h- kin1::kanR leu1:*P_{psy1}*- GFP-psy1*	This study
XLG700	*h- kin1-as1::Gly5-GFP-kanR myo4::ura4+ ade6- leu1-32 ura4-*	This study
XLG709	*h- kin1-as1 leu1:*P_{psy1}*- GFP-psy1 ade6- leu1-32 ura4-*	This study
XLG717	*h- kin1::kanR cps1-191*	This study
XLG724	*h- kin1-as1 GFP-Cfh3:leu1 ade6- leu1-32 ura4-*	This study

Table 2: Mutations in components of the Cell Wall Integrity pathway modulate *kin1*Δ-dependent formation of Cell Wall Deposits. The indicated strains were cultured to mid log phase at 25°C and stained with methyl blue. Percentage of CWD containing cells (mean±SD, n>400) is indicated.

strain	**% of cell with CWD**
WT	None
*kin1*Δ	77 ± 6.5
*kin1*Δ *pmk1*Δ	39.4 ± 2.2
*kin1*Δ *pck2*Δ	17 ± 5.3
*kin1*Δ *rho2*Δ	66.1 ± 0.5
*kin1*Δ *pck1*Δ	91.6 ± 7.8
*kin1*Δ *pmp1*Δ	92 ± 0.9

FIGURE LEGENDS

Fig. 1. Kin1 associates with sterol-rich domains of the plasma membrane and its polarization depends on intact vesicular traffic. A) Kin1 and GFP-Bgs4 localization were determined by sub-cellular fractionation in *GFP-Bgs4* cells and Western blot analysis using anti-Kin1 (upper panels) and anti-GFP (lower panels) antibodies, respectively. Total cell extracts (CE) of exponentially growing GFP-Bgs4 protoplasts were prepared under nondenaturing conditions. Cytosol and membrane fractions were separated by ultracentrifugation. B) Membrane fractions were further separated in buffers containing either 0.5 M NaCl, 100 mM Na_2CO_3 pH 11, 2 M urea, 1% Triton X-100 at 4°C and 2% SDS. C) Brefeldin A (BFA) treatment depolarizes Kin1-GFP on the plasma membrane. *Kin1-GFP* cells were treated for 1h with 100 µg/ml of BFA. Polarization of lipid rafts was assayed by filipin dye as a control for BFA efficiency. Bar, 5 µm.

Fig. 2. Kin1 is required for morphogenesis and cell wall synthesis regulation. A) Wild type (WT) and *kin1*Δ cells were cultured to mid-log phase, fixed at 25°C or after 5h at 37°C and beta-glucans were stained with methyl blue. Arrowheads show the presence of Cell Wall Deposits (CWD) on the lateral cortex. Percentage of CWD containing cells (mean±SD, n>400) are indicated in the top right for *kin1*Δ mutants. Bar, 2 µm. B) *cdc10-V50, cdc25-22,* and *sid4-SA1* mutations (G1 arrest, G2 arrest and septum inhibition, respectively) were combined with *kin1*Δ and compared to *kin1*$^+$ cells (WT). Exponentially growing cells were shifted to 37°C for 4h and stained with methyl blue. CWD are shown by arrowheads. Bar, 2 µm. C) Cell wall structures observed by Transmission Electron Microscopy. Left: the wild type (WT) cell shows cell wall (cw), nucleus (n), plasma membrane (pm), septum (s). Right: *kin1*Δ cells showing two different Cell Wall defects: (a) multiple layers of cell wall, (b)

amorphous cell wall material (am, white arrow) between the plasma membrane and the cell wall. Insets show 2X magnifications of the cell surface. Bars, 1 μm.

Fig. 3. Phenotypic characterization of the inhibition of an ATP analog-sensitive Kin1-as1 kinase. A) Identification of the F220G analog-sensitive (as1) mutation in Kin1 ORF by sequence alignment with Calmodulin Kinase and Cyclin dependent kinase Cdk2 (according to Bishop *et al.*, 2000). B) Effect of Kin1-as1 inhibition on the cell wall and septum: *kin1-as1* cells were incubated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. In the upper panels, cells were stained with methyl blue for fluorescence microscopy (Bar, 2 μm). The asterisk shows an asymmetric septum and arrowheads show CWD. In the middle panels, cells were processed for TEM. Arrows show amorphous cell wall material (am), cell wall (cw), plasma membrane (pm) (Bars, 1 μm). An inset depicts the distinctive nature of the electron density of the cell wall material in the TEM image (Bar, 0.5 μm). The histogram on the lower panel indicates the percentage of *kin1-as1* cells with CWD following incubation with various doses of 1NM-PP1 for 2h at 30°C. C) Localization of Kin1-as1-GFP in cells incubated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. Asterisks depict Kin1-as1-GFP signals on the lateral cortex. The right panel shows a part of the middle image with depolarized Kin1-as1-GFP on the cortex. Bar, 2 μm. D) Mobility of Kin1-as1-GFP protein in the cell end analyzed by Fluorescence Recovery After Photobleaching (FRAP). *Kin1-as1* cells were treated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. Fluorescence of Kin1-as1-GFP in representative cells is shown before the laser bleach (pre), during the bleach (bleach) and at 50 and 100 sec after the bleach (t50 and t100). The bleached areas are indicated by white squares. Bar, 5 μm. The graph on the right shows the mean normalized fluorescence intensity in the cell end plotted over time (n=10 for each condition). The arrow indicates the bleach.

The membrane spanning protein GFP-Psy1 has been processed under identical FRAP conditions as a control.

Fig. 4. Kin1 is required for plasma membrane organization and endocytosis. A) Wild type (WT) and *kin1*Δ cells were stained with filipin. B) GFP-Psy1 localization was detected in exponentially growing WT and *kin1*Δ cells at 25°C. Arrowheads show accumulation of GFP-Psy1 on the lateral cortex. C) FM4-64 (8 μM) incorporation into WT or *kin1*Δ cells was monitored after 1h. D) Localization of GFP-Bgs1 in exponentially growing cells at 25°C was examined in WT and *kin1*Δ strains. Arrowheads denote delocalized GFP-Bgs1 signals. Bars, 2 μm in all micrographs. E) *Kin1*Δ rescues *cps1-191* (*bgs1*^{ts}) sensitivity to heat. Growth of indicated strains was monitored by serial dilutions on YES medium at different temperatures.

Fig. 5. *Kin1-as1* inhibition initially alters growth area polarization and subsequently cell wall structure and Pmk1 activation. *Kin1-as1* cells were treated with either DMSO or 20 μM 1NM-PP1 for 2h at 30°C. A) Cells were stained with filipin to detect sterol-rich domains (Bar, 2 μm), B) GFP-Cfh3 (Bgs1 complex) was localized during different stages of the cell cycle. Arrowheads denote depolarized GFP-Cfh3 on the lateral cortex (Bar, 5 μm), C) Kinetics of CWD formation and GFP-Cfh3 depolarization at the cortex. *Kin1-as1 GFP-Cfh3* cells were incubated with 20 μM 1NM-PP1 and samples were collected at the indicated times. Cfh3 localization was detected by its GFP signal and CWD by methyl blue staining. The percentage of cells in the population is indicated (n>100). D) Pmk1 activation was monitored by western blot. Its activated form was detected using the anti-phospho MAPK antibody (p42/44) in whole cell extracts. Cdc2 was used as a loading control.

Fig. 6. Vesicular trafficking modulates CWD formation. A) Exponentially growing *kin1-as1* cells were treated with Ethanol or Brefeldin A (BFA) for 1h and then DMSO or 20 μM 1NM-PP1 was added for 2h at 30°C. B) Strains of the indicated genotypes were incubated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. Arrowheads show CWD. Samples were stained with methyl blue. Bars, 2 μm.

Fig. 7. The Cell Wall Integrity MAPK pathway is constitutively activated in *kin1*Δ cells and contributes to CWD formation. A) Analysis of CWI MAPK pathway activation in wild type (WT) and indicated mutant cells by Western blot. Whole cell extracts were prepared and Pmk1 activation was monitored using the anti-phospho MAPK antibody (p42/44). Cdc2 was used as a loading control. (B, C) The indicated strains were cultured to mid log phase and growth was assayed by 10-fold serial dilutions on B) YES control plates or YES plates containing 0.1 M or 0.2 M $MgCl_2$, and C) EMM control plates or EMM plates containing the cell wall damaging agents SDS or Calcofluor white (Cw).